Present study investigated the effects of Pinus roxburghii bark ethanol extract (ETHE) in streptozotocin induced diabetic nephropathy in wistar rats. Rats were divided into 5 groups (n = 6). Diabetes was induced with a single dose of streptozotocin (60 mg/kg i.p.). After development of diabetes, the rats were treated with ETHE at the dose of 100, 300 and 500 mg/kg body weight orally for 40 days. The efficacy of ETHE was compared with diabetic control rats. ETHE treatment significantly decreased plasma glucose, creatinine, urea nitrogen, total cholesterol and triglyceride levels. Change in body weight was found to be non-significant in treated groups. It was concluded that ETHE can provide a radical cure for drug-induced diabetic nephropathy by a reduction in renal damage.

ABSTRACT

Present study investigated the effects of Pinus roxburghii bark ethanol extract (ETHE) in streptozotocin induced diabetic nephropathy in wistar rats. Rats were divided into 5 groups (n = 6). Diabetes was induced with a single dose of streptozotocin (60 mg/kg i.p.). After development of diabetes, the rats were treated with ETHE at the dose of 100, 300 and 500 mg/kg body weight orally for 40 days. The efficacy of ETHE was compared with diabetic control rats. ETHE treatment significantly decreased plasma glucose, creatinine, urea nitrogen, total cholesterol and triglyceride levels. Change in body weight was found to be non-significant in treated groups. It was concluded that ETHE can provide a radical cure for drug-induced diabetic nephropathy by a reduction in renal damage.

INTRODUCTION

An ascent in glucose auto-oxidation and hyperglycemia are responsible for micro and macro angiopathy associated with Diabetes mellitus that has been implicated in the induction of an array of diseases including nephropathy[1-2]. Diabetic nephropathy (DN) is one of the most severe and unavoidable microvascular complications of both type 1 and type 2 diabetic mellitus [3]. Various biochemical pathways observed to be included in the pathogenesis of diabetic nephropathy and reactive oxygen species (ROS) appeared to be the shared factor in different pathways. Diabetic nephropathy is characterized by metabolic derangements, systemic and glomerular hypertension, advanced glycation end products (AGEs) and oxidative stress [4]. Moreover, kidney hypertrophy and hyperfiltration with alongside metabolic disturbances prompting the advancement of long-term diabetic renal damage [1].

Pinus roxburghii is a plant known to be a rich in flavonoids [5]. Flavonoids are abundant plant phenolic compounds [6]. Quercetin is one of the most common native flavonoids occurring mainly in glycosidic forms such as rutin [7]. In one of our in-silico study secosiresinol, a constituent reported from Pinus roxburghii indicated aldose reductase enzyme inhibitory activity, [8] which is mainly responsible for diabetic secondary complications [9]. However, the likelihood that Pinus roxburghii could prove beneficial in the amelioration of diabetic renal damage has not been beforehand investigated. Therefore, we decided to evaluate the role of Pinus roxburghii in diabetic nephropathy. Streptozotocin (STZ) induced diabetic nephropathy model on experimental rats was chosen for this study. The impact of ethanol extract of Pinus roxburghii bark (ETHE) on fasting blood glucose level, body weight, kidney function test, serum lipid profile and other biochemical markers connected with diabetic pathogenesis were explored. Finally, a histopathological investigation of kidney was performed to affirm the defensive impact of ethanol extract of Pinus roxburghii bark (ETHE).

MATERIALS AND METHODS

Collection and identification of plant material

The bark of Pinus roxburghii Sarg. were collected from the hilly region of Morni, District Panchkula, Haryana, in the month of February 2013 and was authenticated by Dr. A.K Sharma, Sr. Scientist at Department of Natural Product, FRI, Dehradun, Uttarakhand, India, where a voucher specimen no. 129 FHH was deposited for future reference.

Preparation of extract

Shade dried coarse powdered bark of Pinus roxburghii Sarg. in a quantity sufficient as per the volume of the extractor was packed in a thimble (made of filter paper sheet) and sequentially extracted with petroleum ether, chloroform, ethyl acetate and ethanol. A sufficient volume of solvent was added to the reservoir, and hot continuous extraction process in a Soxhlet extractor was started. This extraction process was continued for about 48 hours or until solvent coming down the siphoning tube became colorless. The over abundance of solvent was distilled under reduced pressure using a rotatory vacuum evaporator. (Heidolph Laborota 4011, digital). Ethanol extract so obtained was stored at 10˚C for further analysis.

Experimental Animal

Male Sprague-Dawley rats (200-250 gm) were used. They were kept at 25 ± 2˚C in a 12 h light, dark cycle with lights on at
07:00h and fed the standard pellet rat diet [Ashirwad Industries, Tirpari, Ropar (Punjab)] and water ad libitum. Institutional Animal Ethics Committee, constituted under the guidelines of CPCSEA, Ministry of Environment, Govt. of India, New Delhi, Approved all the animal experimental protocols (Registration Number: 562/GO/02/a/CPCSEA)

INDUCTION OF DIABETES

Type 1 diabetes was induced in experimental rats by i.p. injection of Streptozotocin (Sigma, St. Louis, Mo., USA) (dissolve in citrate buffer 0.1 mol/L, pH 4.2) in a dose of 60mg/kg body weight for three successive days. Rats were considered diabetic if blood glucose concentration increased up to 200 or more mg/dl [10].

EXPERIMENTAL PROTOCOL

The animals were devided into five groups (06 animals/group) as follows:

Group I: Received vehicle [normal saline: Tween 80 (95:5)] served as normal control group.

Group II: Injected with STZ, i.p. in a dose of 60 mg/kg body weight for 3 successive days and served as diabetic group.

Group III-V: Received ETHE at different doses level of 100, 300 and 500 mg/kg body weight, respectively via oral gavage daily for 40 days, starting after 3 days of STZ injection.

Body weight

The changes in body weight were calculated at the time of STZ treatment and at 40th day of experiment using an automatic electronic balance (A&D Co.Ltd. Japan).

Changes in kidney weight

The weight of the left kidney at sacrifice was measured in grams and was regarded as absolute weight.

Biochemical Analyses

Animals fasted for 24 h fasted animals were sacrificed by cervical decapitication on 40th day of treatment. Trunk blood sample was collected in sample tubes and serum so obtained by centrifugation (at 5000 rpm for 5min) for determination of biochemical parameters. Serum was used for the estimation of glucose, blood urea, albumin and creatinine, serum triglyceride (TG), cholesterol (TC) and high density lipoprotein (HDL). Estimations were carried out as per manufacturer's instruction provided with commercially available kits using auto analyzer (Erba Chem-7, Transasia, New Delhi, India).

Histopathological examination

Kidney removed from the all animal were cleaned and fixed in 10% buffered formalin solution. Then they were embedded in paraffin and stained with hematoxylin-eosin for histopathological studies. All sections were evaluated for the degree of tubular and glomerular injury and necrosis.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by Dennett's "t" tests to determine level of significance using GraphPad InStat version 3.05 for Windows, (GraphPad Software, San Diego California USA). A value of p <0.01 was considered significant results are expressed as mean ± SEM. for six rats in each group.

RESULTS

Changes in body weight in STZ induced diabetic nephropathy

There was marked reduction in the body weight of Group II (diabetic control) as compared to that of the Group I (normal control) and Group III-V (ETHE treated groups) (Table 1). The mean body weight of Group I (normal control) was 195±0.70 gm on 1st day and increased to 220± 0.65 gm at the end of the study. The % increase in body weight of Group I (normal control) was 25%. There was a significant (p<0.01) and sustained decrease in body weight of Group II (diabetic control) 272 ± 0.14 gm on (1st day) and decreased to 107 ± 0.92 gm on (40th day) respectively. Oral administration of ETHE at a dose of 500 mg/kg body weight (Group V) gained significant body weight (65%) in body weight from 218 ± 0.54 gm to 195±0.47 gm at the end of the study.

Changes in kidney weight in STZ induced diabetic nephropathy

There was marked increase in the kidney weight of Group II (diabetic control) as compared to that of the Group I (normal control) and Group III-V (ETHE treated groups) (Table 1). The mean body weight of Group I (normal control) was 0.91±0.19 gm. There was a significant (1.45±0.25 gm) (p<0.01) increase in the kidney weight of Group II (diabetic control). Oral administration of ETHE at a dose of 500 mg/kg body weight (Group V) caused significant improvement (1.04±0.17) in kidney weight of STZ induced diabetic rats.

Table 1. Effect of ETHE on body weight and kidney weight of diabetic rats in STZ induced diabetic nephropathy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Body weight (gm)</th>
<th>% Increase or decrease in body</th>
<th>Kidney weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1st day</td>
<td>40th day</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Normal Control + Vehicle</td>
<td>195 ±0.70</td>
<td>220±0.65**</td>
<td>0.91 ± 0.19**</td>
</tr>
<tr>
<td>II</td>
<td>Diabetic control+ Vehicle</td>
<td>272 ±0.14</td>
<td>107±0.92</td>
<td>1.45 ± 0.25</td>
</tr>
<tr>
<td>III</td>
<td>Diabetic + ETHE (100mg/kg)</td>
<td>208±0.94</td>
<td>143±0.54**</td>
<td>1.30 ± 0.22</td>
</tr>
<tr>
<td>IV</td>
<td>Diabetic + ETHE (300mg/kg)</td>
<td>210±0.57</td>
<td>150±0.77**</td>
<td>1.19±0.07*</td>
</tr>
<tr>
<td>V</td>
<td>Diabetic + ETHE (500mg/kg)</td>
<td>195±0.47</td>
<td>218±0.54**</td>
<td>1.04±0.17**</td>
</tr>
</tbody>
</table>

Values are given as mean±S.E.M (n=6). * p < 0.05 significant from diabetic control animals. ** p < 0.01 significant from diabetic control animals using one-way ANOVA with Dunnett’s t-test. #represent % decrease in body weight, ETHE-ethanol extract of Pinus roxburghii bark

Changes in blood glucose levels in STZ induced diabetic nephropathy

The blood glucose concentration in Group II (diabetic control) increased significantly in comparison to the Group I (normal control) and Group III-V (ETHE treated groups). The mean...
blood glucose level in Group I (normal control) was 120.8±0.60 mg/dl on the 1st day and 93.5±0.78 mg/dl on 40th day (Table 2). There was a significant (p<0.01) and sustained increase in blood glucose level in Group II (diabetic control). In Group II (diabetic control), blood glucose level on the 1st day was 371±0.32 mg/dl and increased to 405.8±0.30 mg/dl on (40th day). Oral administration of ET (ETE) to diabetic rats caused significant decrease in blood glucose level in comparison to the result obtained from Group II. ET at a dose of 500 mg/kg body weight (Group V) caused marked decrease in blood glucose level of diabetic rats. While other test groups III and IV also showed some less significant values of decrease in blood glucose levels (311±0.67 and 293.33±0.82).

Table 2. Effect of ET on Blood glucose level in STZ induced diabetic nephropathy

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Blood glucose level (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st day</td>
<td>40th day</td>
</tr>
<tr>
<td>I</td>
<td>Normal Control + Vehicle</td>
<td>120.8±0.60**</td>
</tr>
<tr>
<td>II</td>
<td>Diabetic control + Vehicle</td>
<td>371.83±0.32</td>
</tr>
<tr>
<td>III</td>
<td>Diabetic + ET (100mg/kg)</td>
<td>396.83±0.19</td>
</tr>
<tr>
<td>IV</td>
<td>Diabetic + ET (300mg/kg)</td>
<td>313.00±0.79</td>
</tr>
<tr>
<td>V</td>
<td>Diabetic + ET (500mg/kg)</td>
<td>340.50±0.39</td>
</tr>
</tbody>
</table>

Values are given as mean±S.E.M (n=6). * p < 0.05 significant from diabetic control animals, ** p < 0.01 significant from diabetic control animals using one-way ANOVA with Dunnett's t-test. ET-ethanol extract of Pinus roxburghii bark

Changes in Serum albumin in STZ induced diabetic nephropathy

Serum albumin excretion was significantly increased in the Group II (diabetic control) as compared to the Group I (normal control) (Table 3). ET in dose of 500mg/kg body weight showed marked reduction in albumin level was 2.36±0.07 mg/dl.

8.4.5 Change in serum urea and creatinine levels in STZ induced diabetic nephropathy

Serum urea and creatinine were significantly elevated in the Group II (diabetic control) as compared to that of Group I (normal control) and Group III-V (ETE treated groups)(Table 3). The mean serum urea level in control animals was 58.98±0.95 mg/dl. There was a significant (p<0.05) and sustained increase in serum urea level of Group II (diabetic control) was 274.08±0.07 mg/dl. The administration of ET to diabetic rats resulted in significant decrease of serum urea level in comparison to that obtained from Group II (diabetic control). Serum urea level of animals treated with ET at dose of 500 mg/kg body weight (Group V) was 67.98±0.51 mg/dl. The mean serum creatinine level in Group I (normal control) was 0.85±0.02 mg/dl. There was a significant (p<0.01) and sustained increase in creatinine level of Group II (diabetic control). Creatinine level of Group V (ET at a dose of 500 mg/kg body weight) decreased significantly to 0.90±0.03 mg/dl.

Changes in lipid Profile in STZ induced diabetic nephropathy

Serum cholesterol and TG level of the Group II (diabetic control) were significantly higher while HDL was lower as compared to the control normal rats. However, administration of ethanol extract Pinus roxburghii bark (ETE) significantly improved these parameters in a dose-dependent manner as shown in Table 3.

Table 3. Effect of ET on biochemical parameters in STZ induced diabetic nephropathy

<table>
<thead>
<tr>
<th>Parameters /Groups</th>
<th>I (Normal Control + Vehicle)</th>
<th>II (Diabetic control + Vehicle)</th>
<th>III (Diabetic + ETHE (100mg/kg))</th>
<th>IV (Diabetic + ETHE (300mg/kg))</th>
<th>V (Diabetic + ETHE (500mg/kg))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>8.45±0.02**</td>
<td>85.82±3.82*</td>
<td>72.10±1.28*</td>
<td>67.98±0.51*</td>
<td></td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>3.07±0.06</td>
<td>54.50±2.56</td>
<td>48.66±2.81*</td>
<td>48.16±1.35*</td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>2.69±0.09</td>
<td>54.50±2.56</td>
<td>48.66±2.81*</td>
<td>48.16±1.35*</td>
<td></td>
</tr>
<tr>
<td>Serum triglyceride (mg/dl)</td>
<td>23.16±1.33</td>
<td>75.60±4.70</td>
<td>28.16±2.07*</td>
<td>23.50±0.76*</td>
<td></td>
</tr>
<tr>
<td>Serum hdl (mg/dl)</td>
<td>10.50±0.50*</td>
<td>4.16±0.70</td>
<td>7.50±0.70*</td>
<td>8.30±0.61**</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean±S.E.M (n=6). * p < 0.05 significant from diabetic control animals, ** p < 0.01 significant from diabetic control animals using one-way ANOVA with Dunnett's t-test.

Histological Changes in renal tissue in STZ induced diabetic nephropathy

Histological examination of control and ethanol extract Pinus roxburghii bark (ETE) treated groups showed normal cells structure of kidney. STZ treatment elicited significant morphological changes in kidney of diabetic group rats with severe renal injury. Kidneys of control animals showed spherical shape cells with granulated cytoplasm (Figure 1 a&b) but in kidney of diabetic group animals showed swelling, ruptured cell wall and distorted cytoplasm with increased vacuolation (Figure 1 c&d). These changes were significantly inhibited in ethanol extract Pinus roxburghii bark (ETE) treated groups (300 or 500 mg/kg body weight). Treatment of diabetic animals with ethanol extract Pinus roxburghii bark (ETE) (300 or 500 mg/kg body weight) significantly caused preventive effect on cells i.e. cell wall regeneration with less vacuoles size and granular cytoplasm (Figure 1 g&h & i&j).
Figure 1 (a & b): Photomicrograph showing kidney cells of control animal stained with haematoxylin and eosin healthy cells having spherical shape and granulated cytoplasm. X 1000. Fig. 1 (c&d): Photomicrograph showing diabetic group animal kidney cells stained with haematoxylin and eosin showing swelling, ruptured cell wall and distorted cytoplasm with increased vacuoles. X1000. Fig.1 (e&f): Photomicrograph showing kidney cells treated with 100 mg/kg body weight of ETHE stained with haematoxylin and eosin showing less swelling, regeneration of cell wall with decrease in vacuoles size. X1000 Fig.1(g&h): Photomicrograph showing kidney cells treated with 300 mg/kg body weight of ETHE stained with haematoxylin and eosin showing intermediate preventive effect showing circular and oval shaped cells, less granular cytoplasm, regeneration of cell wall with less vacuoles size. 15X x 40X. Fig.1 (i&j): Photomicrograph showing kidney cells treated with 500 mg/kg body weight of ETHE stained with haematoxylin and eosin showing maximum preventive effect showing oval shaped cells, granular cytoplasm, regenerated cell wall and intermediate vacuoles. 15X x 40X
DISCUSSION

Diabetes induced by streptozotocin (STZ) causes the pulverization of beta cells of islets of Langerhans [11] which prompts the reduction in insulin discharge. An insufficient release of insulin causes high blood glucose level specifically hyperglycemia, which results being developed of diabetic complications (4) because of oxidative damage cause by the generation of reactive oxygen species (ROS) [12].

Blood glucose level in diabetic rats was critical basal parameters for observing diabetes.[10] Ethanol extract Pinus roxburghii bark (ETHE) in various dosages indicated critical lessening in blood glucose. These finding proposes that ethanol extract Pinus roxburghii bark (ETHE) may enhanced the disturbed metabolism associated with diabetes.

Induction of diabetes with STZ is connected with the trademark loss of body weight and additionally increases in the kidney weight [14]. Our study showed that there was marked decrease in the aggregate body weight and additionally increase in the kidney weight of the diabetic group when compared with that of the control group. Ethanol extract Pinus roxburghii bark (ETHE) treatment demonstrated a noteworthy enhancement in kidney weight and body weight in a dose dependent manner.

In Diabetic nephropathy, significantly higher plasma concentration of triglyceride (TG) but lower level of high density lipoprotein (HDL) was found. [15] The study showed that serum level of glucose, serum cholesterol, and triglyceride (TG) were significantly high in diabetic rats as compared to that of the normal control rats. Administration of ethanol extract Pinus roxburghii bark (ETHE) altogether enhanced the level of serum glucose, serum cholesterol and triglyceride (TG) parameters in a dose-dependent manner. In addition, the highest dose of extract (500 mg/kg) was able to reduce the blood glucose to normal level. The serum level of high density lipoprotein (HDL), which was significantly decreased in diabetic rats, was also improved by ethanol extract Pinus roxburghii bark (ETHE) in a dose-dependent manner. These findings may indicate that ethanol extract Pinus roxburghii bark (ETHE) can improve the lipid profile of diabetic rats.

In addition, results demonstrated that serum urea and creatinine level were altogether raised in diabetic group animals as compared to that of the control normal rats. Serum urea declined significantly in the ethanol extract Pinus roxburghii bark (ETHE) treated groups, however, the level of creatinine improved only with the highest dose of ethanol extract Pinus roxburghii bark (ETHE). Serum urea corrected to normal levels in the group treated with the highest dose of ethanol extract Pinus roxburghii bark (ETHE) (500 mg/kg body weight). Moreover, albumin (a marker of early Diabetic nephropathy [16] was improved after treatment with ethanol extract Pinus roxburghii bark (ETHE) in a dose-dependent manner.

This information may demonstrate that the defensive impact of ethanol extract Pinus roxburghii bark (ETHE) against renal damage in diabetic rats is dose-dependent. These outcomes demonstrated that ethanol extract Pinus roxburghii bark (ETHE) forestall renal damage in Diabetic nephropathy (appeared in histopathological results).

A variety of phenolic and flavonoids have been purified from Pinus roxburghii demonstrates capacity to restrain nitric oxide (NO) or reactive oxygen species (ROS) production[17]. Phenolic compounds are very useful in oxidative stress and chronic inflammation as they remove reactive oxygen species (ROS) by their free radical scavenging mechanism[18]. Novel bioactive flavonoid compounds are presently in assessment for combating many oxidative stress-related complications such as cancer, diabetes, arthritis, alzheimer’s and parkinson’s[19]. The present results recommend that ethanol extract Pinus roxburghii bark (ETHE) show critical hypoglycemic, hypolipidemic and nephroprotective impacts in STZ induced diabetic rats.

CONCLUSION

The present study demonstrated that ethanol extract Pinus roxburghii bark (ETHE) has the ability to secure kidney damage, thus attenuating Diabetic nephropathy in Wistar rats. The biochemical results were additionally in agreement to our histological discoveries that the diabetic rats treated with ethanol extract Pinus roxburghii bark (ETHE) indicated negligible changes, in comparison to that of the normal control rats. Thus it can be inferred that the ethanol extract Pinus roxburghii bark (ETHE) had nephroprotective potential, making it a plant to be utilized as a part of treatment of Diabetic nephropathy.

REFERENCES


